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Title: Thermal refugia and the survival of species in changing environments: new evidence from a nationally extinct freshwater fish.

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RUNNING HEAD: Burbot colonisation of English rivers

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ABSTRACT

Variation in global climate during the Quaternary has helped shape current species distributions. The stenohaline fish fauna of the British Isles is generally thought to have colonised eastern England via a landbridge following the last glacial maximum. This theory is investigated using the nationally extinct burbot, *Lota lota*, as a model species. Samples were collected from 15 museum specimens of known English provenance and analysed for differences in the mitochondrial DNA control region. The DNA analysis produced eight sequences of 270 basepairs, with one sample reaching 420 basepairs in length. Genetic analysis suggests the extinct English population of the burbot was a distinct lineage, differing from those previously described from across the species' global distribution. Despite this, network analysis suggests that the English lineage is closely related to populations in western Europe, supporting colonisation via a post-glacial landbridge. The rate of genetic divergence suggests that the timing of *L. lota*'s colonisation of English rivers was prior to the last glacial maximum. *Lota lota* appears to have survived the last glacial maximum in refugia within the British Isles. This study adds to the evidence for a British freshwater refugia and furthers our understanding of the colonisation history of British freshwater fishes. These results also provide valuable information for conservation strategies for *L. lota* indicating the western European clade as most genetically appropriate for potential future reintroductions to English rivers.

INTRODUCTION

From the beginning of the Quaternary (2.6 million years before present, ybp) and the formation of the Arctic ice cap to the present day, the Northern Hemisphere has been subjected to cyclical patterns of ice sheet expansion and contraction (Hewitt 2000) which has dramatically influenced the distribution and genetic structure of the current biota (Hewitt 1996; Dynesius and Jansson 2000). During the Quaternary glacial periods, temperate regions of the Northern Hemisphere saw extinctions and southward shifts in species ranges, with a corresponding recolonisation into northern areas during the warmer interglacials (Culling et al. 2006). Phylogeographical analysis has suggested many western European species persisted during these glacial periods in refugia located in Iberia, Italy, the Balkans-Greece and the Caspian/Caucasus regions (Hewitt 2004). Terrestrial post-glacial colonisation patterns have been described based on several model species (Hewitt 1999), with geographical features such as mountain ranges and water bodies impacting species' capacity to disperse (Taberlet et al. 1998). Freshwater fishes, however, are restricted in their dispersal ability by the interconnectedness of river catchments (Culling et al. 2006; Reyjol et al. 2007) leading Hewitt (2004) to suggest a 'new paradigm' was required. Post-glacial colonisation of European rivers appears to have been generally from the Black Sea via rivers such as the Danube or Dnieper, although routes and glacial refugia are often species specific (Hewitt 2004; Makhrov and Bolotov 2006). More recent studies (e.g., Hänfling et al. 2002; Finnegan et al. 2013) have suggested some species may have persisted in 'cryptic northern refugia' (Stewart and Lister 2001).

In his paper, 'The origin and distribution of the freshwater fishes of the British Isles', Wheeler (1977) discussed two potential mechanisms for the colonisation of British rivers by stenohaline fishes. The first, proposed by (Scharff 1899) was that the freshwater fish species

of southern and eastern England colonised following the last glacial maximum during the existence of a land bridge between Great Britain and Europe. The land bridge is thought to have persisted until 7500 ybp, enabling the connection of the eastern English rivers from the Humber through East Anglia, with the Rhine system as tributaries or through the formation of a shared seasonal floodplain (Wheeler 1977). The second, proposed by Orkin (in Schindler 1957), was that certain species persisted in refugia during the last glacial maximum, having established in British rivers during an earlier interglacial. Wheeler (1977) suggests that this glacial refugia theory is unlikely, due to the unsuitable environmental conditions for spawning, although he did advocate the possibility that both mechanisms may have been possible. The landbridge theory would seem to be supported by the present day distribution of freshwater fishes as species richness is reduced to the north and west of the British Isles (Maitland and Lyle 1996), although this pattern is confused by human induced movements of fishes or dispersal through canal systems (Wheeler and Easton 1978).

The distribution of the freshwater gadoid, the burbot *Lota lota*, stretches across North America and Eurasia (Scott and Crossman 1973). Despite this extensive range many populations, particularly in western Europe, are threatened with extirpation (Stapanian et al. 2010), while *L. lota* is thought to have become extinct in English rivers in the early 1970s (Worthington et al. 2010). Recent analysis of mitochondrial DNA sequence variation in *L. lota* from across its global distribution suggested division into two sub-species; *L. l. lota* found in Eurasia and Alaska and *L. l. maculosa* from North America, south of the Great Slave Lake (Van Houdt et al. 2005). After separation, the *L. l. lota* population was limited to a glacial refuge from where it colonised Eurasia (Van Houdt et al. 2003). European *L. lota* have been subjected to three or four subsequent glacial cycles, splitting the species into several genetic clades (see Fig. 1 in Van Houdt et al. 2005). It is hypothesized that the English

population belonged to the western European clade owing to the likely colonisation of English rivers via the landbridge that existed following the last glacial maximum. *Lota lota*'s former English distribution corresponds well with this theory, as the species was restricted to forty-two rivers in eastern England prior to extinction around the beginning of the 1970s (Worthington et al. 2010).

The aim of this study was to examine the origin and timing of the colonisation of the British stenohaline fish fauna using *L. lota*. Due to its recent extirpation from English rivers, *L. lota* is subject to an investigation as to the feasibility of reintroduction to the rivers of its former English range (Worthington et al. 2009). As such, understanding the genetic relationship between the former English *L. lota* population and potential source populations is an important consideration in reintroduction planning (Leonard 2008).

MATERIAL AND METHODS

SAMPLES

Tissue samples were collected from *L. lota* specimens known to have been captured in England. Museums and universities with natural history collections were contacted to determine whether they held preserved English *L. lota*, and samples were taken from those with suitable specimens (Table 1). A sample of accessible material, either fin clips or muscle tissue, was collected either by the institution's staff or a trained taxidermist. The samples were either from dried taxidermy specimens (n = 8) or fixed using formaldehyde and stored using Industrial Methylated Spirit (IMS, n = 7). The English samples were then compared to sequences from burbot collected from across the species' global distribution (GenBank accession numbers AY656840–AY656915; Van Houdt et al. 2005).

DNA EXTRACTION AND SEQUENCE ANALYSES

DNA analyses were carried out in a laboratory dedicated to the analyses of archival material. Burbot material had never previously been sequenced within the building that housed the laboratory, removing the possibility of contamination from samples outside the study. To control for cross sample contamination, analyses were duplicated with a maximum of three samples analysed concurrently. All sequencing was carried out in a laminar flow cabinet. DNA was extracted using the NucleoSpin Extraction kit (Machery-Nagel GmbH). The digestion, undertaken over a period of 3-5 hours, was enhanced by grinding the samples with a pestle during the incubation phase, for larger tissue samples a double digestion volume was used when required. The DNA from a single spin column was eluted twice with 50 µl of heated elution buffer and stored separately. DNA quality was assessed by means of agarose gel electrophoresis.

Glacial lineages of *L. lota* have been described using Domain I, 450 basepairs (bp), of the mitochondrial control region (CR) which has been identified as containing 90% of the variation found within the entire *L. lota* control region (Van Houdt et al. 2005). Domain I of the CR was targeted using the primers L19ProGm (5'-CCACTAGCTCCCAAAGCTAGA-3') and HDL400L1 (5'-GATTTAGGATTTATGTACTCC-3') resulting in an amplicon of approximately 420 bp (Van Houdt et al. 2005). The L19ProGm primer was also combined with the newly developed HDL230L1 primer (5'-CGCTAGATGATCTCTTACTAC-3') specifically to amplify the first 270 bp of the CR. A minimum of two independent PCR amplifications were carried out per sample per marker. The PCR was carried out in 30 µl containing 1x PCR buffer (Invitrogen), 1-20 ng template DNA, 2.0 mM MgCl₂, 0.2 mM dNTPs, 0.6 units of Taq DNA polymerase (Platinum *Taq*, Invitrogen), and 0.4 µM of forward and reverse primer. The PCR profiling commenced with an initial denaturation of 3 minutes

at 94 °C, followed by 40 cycles of 45 seconds at 94 °C, 50 °C and 72 °C, finishing with a final 7 minutes at 72 °C.

PCR products were purified by means of the Nucleofast PCR cleanup (Machery Nagel, GmbH) or with “GFX PCR DNA and Gel Band Purification kit” (GE Healthcare). Cleaned PCR products were sequenced in both directions using the BigDye version 3.1 cycle sequencing kit (Applied Biosystems) on an ABI 3100. Sequences were analyzed and assembled with SeqScape version 2.5 (Applied Biosystems).

NETWORK ANALYSES

A statistical parsimony (SP) network (Templeton et al. 1992) using TCS version 1.3 (Clement et al. 2000) was built to map the genetic relationship of the former English population to the global distribution developed by Van Houdt et al. (2005). Network version 4.0.0.1 (<http://www.fluxus-engineering.com>) was used to construct reduced median (RM) and median joining (MJ) networks (Bandelt et al. 1999; Bandelt et al. 2000). To estimate the divergence time between English and Continental haplotypes reduced median-joining networks were used to calculate $q \pm r$ (Forster et al. 1996), where q is the average distance from all descendant haplotypes to the ancestral node of the median-joining network and r is a variance estimator. The q statistic was translated into years using a mutation rate for the *Lota* *lota* control region of 2-6% per million years. This value was based on previous research estimating lineage specific mutation rates (Van Houdt et al. 2005). The absolute time since divergence estimations should be interpreted with caution; however, the rough scale of time since divergence can provide valuable insights.

RESULTS

SAMPLES

A total of fifteen *L. lota* specimens were sampled (Table 1), three of which had duplicate samples taken (BUR05, BUR10 and BUR15). The samples were almost exclusively from the River Trent (6 samples) or Great Ouse (8) catchments with only a single sample from Yorkshire in the northern extent of the species' former English distribution (Fig. 1).

DNA EXTRACTION AND SEQUENCE ANALYSES

Two DNA extracts were obtained for each of the 15 *L. lota* specimens (n = 36, extraction was duplicated for specimens BUR05, BUR10 and BUR15). A 420 bp amplicon was achieved for a single specimen (BUR05, both samples), however 270 bp amplicons were obtained for eight out of the 15 specimens and no sequence was obtained for the remaining seven specimens (Table 1; GenBank accession numbers KJ381202-KJ381212). Samples that had been taken from the taxidermy specimens more frequently produced sequences (6 sequences from 8 specimens) than those stored in IMS (2/7). For quality control, the obtained sequences were compared to the mitochondrial genome sequence of *L. lota* (AP004412).

In comparison to the reference sample, three C->T mutations at positions 15718, 15719 and 15892 were observed in a single sample for the BUR10 specimen. These mutations, however, were absent from the second BUR10 sample. A C->T variant at position 15785 was observed in BUR07 that was not present in any other specimen and a replicate sample was not available to confirm this mutation. Consequently, Both BUR10 and BUR07 were removed from further analyses. All other variants were observed in at least two independent specimens or the matching samples from the same specimen. Overall, one new English haplotype was detected in the 270 bp amplicon: a haplotype shared by five specimens (BUR01, BUR02, BUR05, BUR06, and BUR15) from the Rivers Trent, Wissey, and Tame. All specimens were

characterized by an A->G mutation except BUR11 which was similar to the reference sequence at that position. In the specimen from which a 420 bp sequence was obtained (BUR05), two additional T->C transitions at 16031 and 16081 were observed (positions 330 and 380 on the 420 bp amplicon). These variants were confirmed in the two independently analyzed samples of this specimen.

NETWORK ANALYSES

Separate network analyses were constructed for both the 270 bp and 420 bp sequences. Firstly, for the 420 bp sequence that combined the BUR05 haplotype with the relevant known European haplotypes. All network construction methods produced identical results (Fig. 2a). The BUR05 haplotype was clearly different from the previously reported *L. lota* haplotypes, differing by two mutations at positions 144 and 381 from EB01, a variant observed in western Europe. The same analysis was performed on the 270 bp sequence, including the BUR11 haplotype. Due to the limited dataset, the BUR11 haplotype could not be distinguished from the common Eurasian/West European haplotype (Fig. 2b). Nevertheless, the shared BUR01, BUR02, BUR05, BUR06, BUR15 haplotype was still differentiated by a single mutation from all other known variants. Using a divergence estimate of 2-6% per million years to examine the observed divergence in the 420 bp and 270 bp data sets, indicates that the English population diverged from the continental population between 80,000 and 240,000 years ago for the 420 bp data set and between 62,000 and 186,000 year ago for the 270 bp data set.

DISCUSSION

Analysis of the 420 bp sequence from BUR05 suggests that the extinct English *L. lota* population was distinct from the clades highlighted by Van Houdt et al. (2003; 2005) who

sampled *L. lota* from across its global distribution. In this data set we find the presence of a haplotype in England that is not present in the western/northern Europe samples examined by Van Houdt et al. (2005) which included 84 samples from across the species range in western/northern Europe and yielded 13 distinct haplotypes. Given the sampling effort of Van Houdt et al. (2005), the absence of the English haplotype from western/northern Europe suggests that the English haplotype is indeed restricted to England and not the result of a sampling artifact.

The six useable samples produced two new English haplotypes in the 270 bp amplicon data set. The 270 bp network provides additional insight about geographic differentiation between England and western/northern Europe. The relationships among these shorter haplotypes illustrates a single shared haplotype with western/northern Europe while none of the other English samples group within any of the other geographic areas; further suggesting a distinct English lineage of *L. lota*. While the results suggest a slight divergence of English *L. lota*, the analysis reveals the extirpated population was closely related to western and central European clades and likely founded through colonisation from western Europe via a land bridge (see Wheeler 1977). Historical literature published from the 12th century onwards suggests that *L. lota* was confined to rivers of eastern England (Worthington et al. 2010), which would have been linked to the Rhine system as tributaries or through the formation of a shared seasonal floodplain (Wheeler 1977). The sequences from the English population share much of the genetic code with the haplotypes of the central and western European clades including the ancestral EB30 from central Europe. However, degradation of the samples, owing to age and storage, meant that with one exception, too few base pairs were produced to fully disentangle the relationship between former English population and these two ancestral clades. The sole exception permitted a preliminary evaluation of the relationship, which suggests the English

population diverged from the western European clade. However, this result should be treated with caution due to the lack of replication.

Despite supporting the mainland European origin of *L. lota* in the British Isles, the presence of a distinct haplotype provides evidence that *L. lota* was present in England during the last glacial maximum (~14,000 ybp). Our study gives further support to the hypothesis that certain species survived recent glaciations in northern refuges (Stewart and Lister 2001). The freshwater fish fauna of Great Britain is generally considered to be comparatively young in geographical and evolutionary terms (Hughes et al. 2001), with the widely accepted theory being that stenohaline fishes colonised from the Rhine basin following the last glacial maximum (see Wheeler 1977). However, species with current northerly distributions (> 60°N) appear to have ecological and physiological traits that would have allowed them to persist in northern glacial refuges (Bhagwat and Willis 2008). The physiological tolerance of *L. lota* would help to explain the possibility the species survived in English rivers during the Devensian (12,000 – 110, 000 ybp). The extent of the ice sheet during the last glacial maximum indicates a significant proportion of the *L. lota*'s English distribution would have been unavailable (Bowen et al. 2002; Fig. 1). However, *L. lota* are cold adapted (Hölker et al. 2004) and able to spawn in temperatures as low as 1°C (McPhail and Paragamian 2000 and references therein). Evidence from the archaeological record also supports the presence of *L. lota* prior to the last glacial maximum, with the species recorded from lower Palaeolithic (300,000 – 2.5 million ypb) deposits at Barnham, Suffolk (Ashton et al. 1994). This study and similar phylogenetic analyses for other cold-adapted taxa, bullhead *Cottus gobio* (Hänfling et al. 2002) and brown trout *Salmo trutta* (García-Marín et al. 1999; McKeown et al. 2010), suggest that freshwater fishes may have colonised the British Isles prior to and persisted through the last glacial maximum.

270

271 The study was based on samples collected from fifteen specimens, of which eight provided
272 suitable material for sequencing. The samples analysed are thought to represent the majority
273 of available museum *L. lota* specimens of known English origin (pers. obs.). Analysis of
274 historical material stored in museum collections is one of the only ways to map genetic
275 relationships for extinct populations (e.g., Hammond et al. 2001; Gugolz et al. 2008). As
276 such, analyses consisting of limited samples still provide valuable insight for understanding
277 phylogenetic and conservation questions (e.g., reintroduction; Pages et al. 2009). Future
278 genetic analysis could include data from bone fragments, with those from outside the species'
279 known range (e.g., the River Thames; Astill and Lobb 1989; Hawkes and Fasham 1997)
280 potentially providing greater evidence for a glacial refugium. While this study is underpinned
281 by a modest sample size in the context of modern genetic investigations, the authors consider
282 that the results provide a preliminary picture of the relatedness of the former English stock to
283 the remainder of the global population. The validity of the results is underlined by replicated
284 sequences from two different samples from the same specimen and the close correspondence
285 between sequences from geographically separate river catchments.

286

287 This study suggests that the extirpated English *L. lota* was genetically different from the other
288 populations in both Eurasia and North America. Despite this difference, the English
289 population was closely related and probably diverged from those in central and western
290 Europe. This provides a framework for selecting suitable source populations, should
291 reintroduction of *L. lota* to English rivers be deemed feasible. There appears the possibility
292 that *Lota lota* colonised English rivers prior to the last glacial maximum surviving the ice age
293 in refugia. This information, together with studies of other freshwater fishes, illustrates that

the impact of global climatic cycles on species distributions are species specific and linked to the organism's physiological, biological and ecological traits.

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420 **TABLES**

421 Table 1: The capture date and location of the fifteen *Lota lota* specimens if known, the current location of the specimen and the success of the
422 genetic analysis.

Ref	River	Location	Date	Institution	Preservation	Sequenced	GenBank accession numbers
BUR01	River Trent	nr Nottingham	3 Mar 1905	Wollaton Park Museum	Dried	Yes	KJ381202
BUR02	River Trent	-	-	Wollaton Park Museum	Dried	Yes	KJ381203
BUR03	Old West River	Nr. Aldreth Bridge	21/22 Sep 1969	Cambridge University Museum	IMS	No	
BUR04	Great Ouse	Welney	1929	Cambridge University Museum	IMS	No	
BUR05	River Wissey	-	1936	Norwich Museum	Dried	Yes	KJ381204-5
BUR06	River Tame	-	1886	Warrington Museum	Dried	Yes	KJ381206
BUR07	River Thet	-	-	Victoria Museum, Cawthorne	Dried	Yes	KJ381207
BUR08	River Trent	-	-	Calke Abbey	Dried	No	
BUR09	River Trent	-	-	Calke Abbey	Dried	No	

BUR10	Middle Level Drain	-	1884	Wisbech Museum	Dried	Yes	KJ381208-9
BUR11	River Thet	Thetford, Norfolk	-	Natural History Museum	IMS	Yes	KJ381210
BUR12	-	Yorkshire	-	Natural History Museum	IMS	No	
BUR13	River Thet	Thetford, Norfolk	-	Natural History Museum	IMS	No	
BUR14	-	Nr. Cambridge	-	Natural History Museum	IMS	No	
BUR15	River Trent	East Stockwith	3 Nov 1869	Oxford University Museum	IMS	Yes	KJ381211-12

FIGURE CAPTIONS

Fig. 1. The rivers of the former English *Lota lota* distribution in relation to the maximum ice sheet extent during the last glacial maximum (red dashed line, adapted from Bowen *et al.*, 2002). Location of samples with specimen capture site and river information denoted by green circles (numbers relate to sample number in Table I), rivers/areas without site information marked.

Fig. 2. Reduced median-joining network of *Lota lota* control region haplotypes for (a) the 420 bp data set and (b) the 270 bp data set showing the relationship between the English haplotypes (green circles) and haplotypes from Alaska (yellow), Beringia (pink), Eurasia (red), West Europe (blue), and North Europe (purple). Each branch represents a single nucleotide change and black dots indicate unsampled haplotypes.

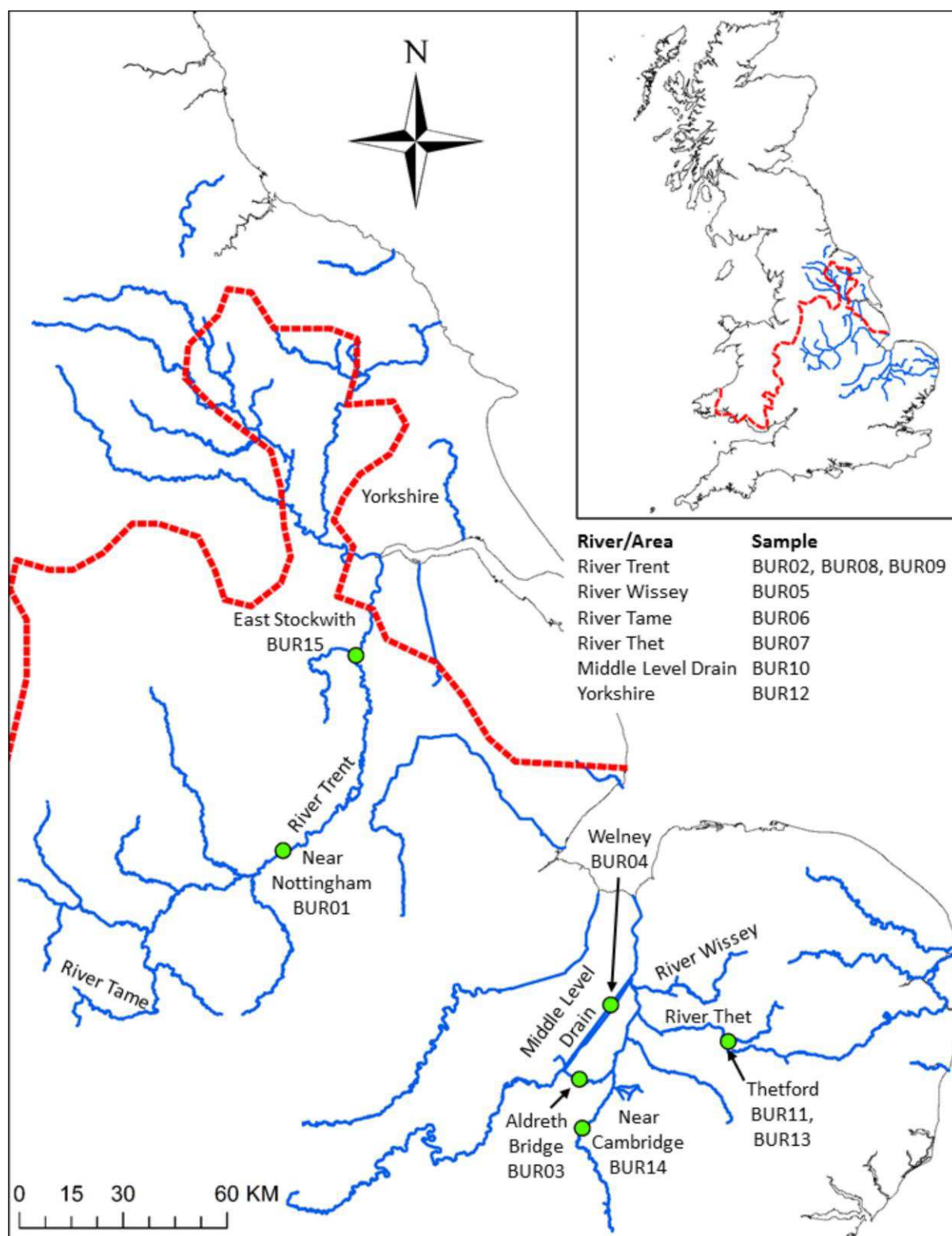
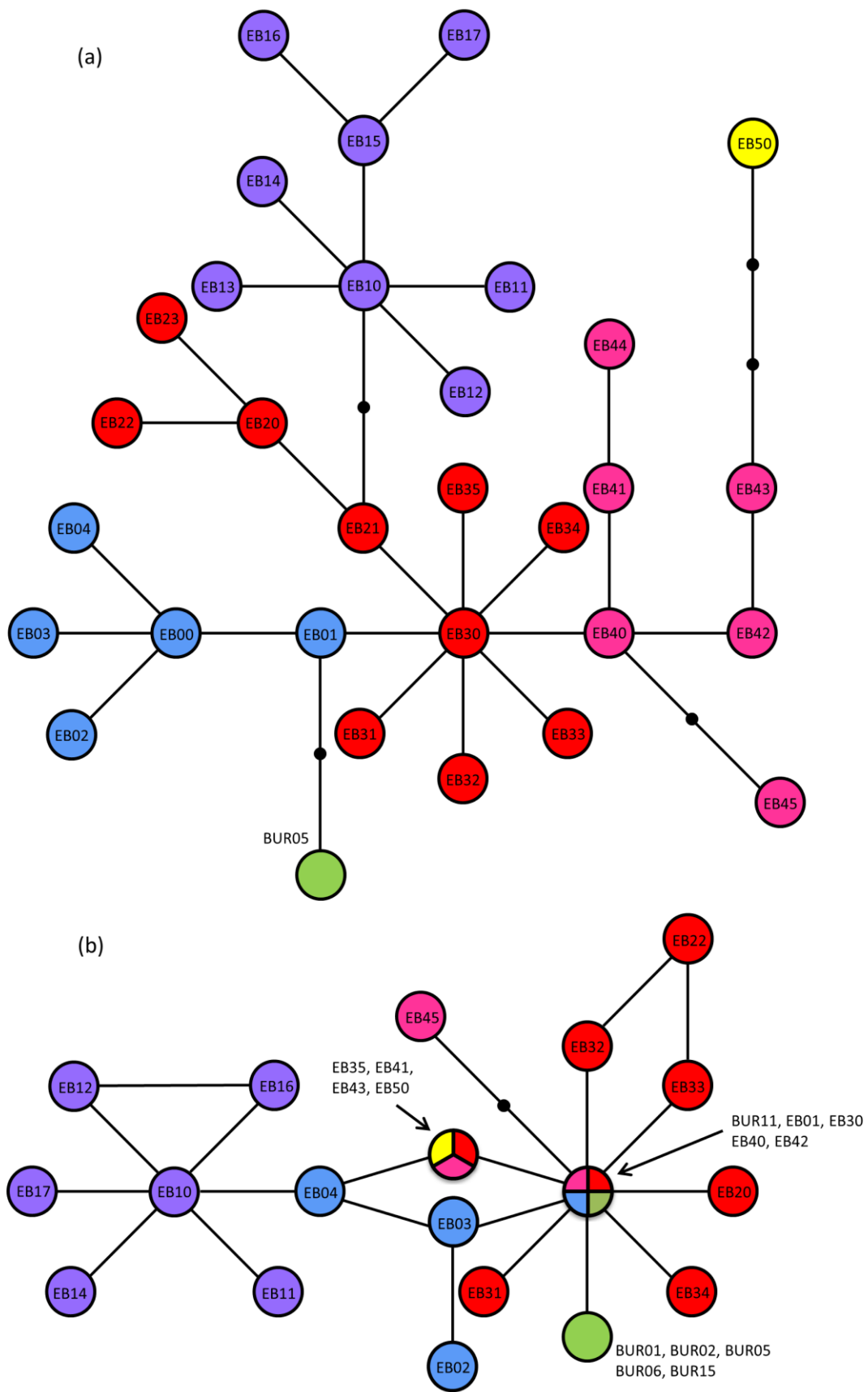


Fig. 1.



440

441 Fig. 2.